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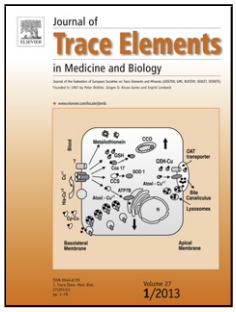

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Differential cytolocation and functional assays of the two major SLC30A8 (ZnT8) isoforms

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Highlights:

- Two isoforms (splice variants) of ZnT8 differ in subcellular localization in human cells.
- Surface localization of ZnT8 in *X. laevis* oocytes allowed developing a ^{65}Zn transport assay.
- The amino acid at position 325, responsible for the R and W variants, is poorly conserved.
- Differential localization of isoforms may be important for ZnT8 function and antigenicity

Abstract. The non-synonymous single nucleotide polymorphism (SNP) rs13266634 in human zinc transporter 8, ZnT8 (SLC30A8), leads to a R325 variant, which is associated with an increased risk of developing Type 2 Diabetes (T2D). Although the molecular details remain unknown, the mutation is thought to alter the kinetics of zinc transport into insulin granules in pancreatic β-cells. Nevertheless, analysis of ZnT8 sequences from several animals shows that the amino acid at position 325 is poorly conserved. Apart from this particular SNP,

human ZnT8 also has two isoforms (splice variants) that differ in length regarding a 49 amino acid N-terminal extension. When expressed in human embryonic kidney (HEK293) cells, the long isoform was present in the plasma membrane in addition to internal membranes, whereas the short isoform was localized mostly to internal membranes. Our observation that human ZnT8 variants and isoforms expressed in *Xenopus laevis* oocytes are all localized at the cell surface allowed us to develop a zinc transport assay using the radioactive isotope ^{65}Zn. We found no detectable differences in zinc transport between W and R variants and no statistically significant differences between long and short isoforms of the W325 variant. Our findings of different cytolocation of ZnT8 isoforms could be relevant for β-cell zinc metabolism in health and disease.

Keywords: Zinc, vesicular CDF transporters, ZnT8, β-cell insulin granules, diabetes

Introduction

Zinc is a micronutrient that is essential for virtually all biological processes [1]. Acquisition, storage, and efflux of zinc are orchestrated by transporters belonging to two families: the Zrt/Irt-like Transporter (ZIP; also known as SLC39A) family, which mediates the flux of zinc from the extracellular environment or from the lumen of organelles into the cytosol, and the Zinc Transporter (ZnT; also known as SLC30A) family, which is part of the larger Cation Diffusion Facilitator (CDF) family and mediates the flux of the metal ions away from the cytosol [2, 3]. The concerted actions of these transporters are a prerequisite for the control of zinc homeostasis. Dysfunctional ZIP or CDF members lead to disorders, such as Acrodermatitis Enteropathica, transient neonatal zinc deficiency, Spondylocheirodysplastic Ehlers-Danlos Syndrome, Type 2 Diabetes (T2D), and many others [4, 5].

Zinc transporter 8 (ZnT8) is a member of the CDF family of proteins and expressed almost exclusively in pancreatic α- and β-cells [6-8] and in kidneys [4, 9]. In pancreatic β-cells, ZnT8 transports zinc from the cytosol into the lumen of insulin granules [10], where zinc assists insulin packaging into hexamers with two bound zinc ions and one bound calcium ion. Besides being located mainly in the membrane of insulin granules in β-cells, ZnT8 is also observed in the plasma membrane and in its proximity in the β-cell-derived INS-1E cell line

[11, 12]. Each functional ZnT8 transporter molecule is a dimer of two identical monomers. Each monomer has six transmembrane domains with N- and C-termini facing the cytosol. The C-terminal domain has a metallochaperone-like fold. There are two splice variants (isoforms) of human ZnT8 as shown by quantification of their respective transcripts in an RNAseq analysis of human pancreatic islets [13]. These isoforms differ at the N-terminus, where one of them has a 49-amino acid extension. The longer isoform (Q8IWU4_1) has 369 amino acid residues and the shorter isoform (Q8IWU4_2) has 320, with relative transcript abundances of about 70% and 30%, respectively [13]. The majority of the studies so far have focused on the longer isoform. Whether or not the two isoforms differ functionally is not known.

Genome-wide association studies (GWAS) showed that carriers with the non-synonymous single nucleotide polymorphism (SNP) rs13266634 in human *ZnT8* have an increased risk of developing T2D [14-16]. This SNP results in an arginine (R) instead of a tryptophan (W) at position 325 in the C-terminus (numbering for the long isoform). The functional association of R325 with an increased risk of T2D is unclear. One hypothesis is that ZnT8 carrying R325 has altered zinc transport activity that affects the synthesis, storage, and/or secretion of insulin. Fluorescence- and radioactivity-based studies on the rodent pancreatic β-cell lines MIN6 and INS-1E overexpressing W or R variants of ZnT8 showed lower transport activity of the R325 variant [7, 17]. In contrast, a recent study found the R325 variant to be more active, both in HEK293 cells and when incorporated into an artificial proteoliposome bilayer [18]. These opposite outcomes likely reflect conditionality of ZnT8 function, and whilst these experimental models are useful, they have drawbacks in the interpretation of the results. It is virtually impossible to control transport conditions in transfected cells where ZnT8 moves zinc ions into vesicles. In contrast, experimental conditions can be tightly controlled in transport assays utilizing proteoliposomes, but such a system is highly artificial when compared to a cellular environment. The conditional K_m value of ZnT8 for Zn^{2+} (>100 µM) in artificial proteoliposomes [18] is at least 250,000 times higher than the resting cytosolic [Zn^{2+}] in β-cells [19]. Therefore, there is a need to address the ZnT8 structure-function relationships in alternative assay systems.

The main objective of the present study was to establish a cell-based zinc transport assay that would allow comparing the kinetic properties of human ZnT8 variants and

isoforms. Heterologous expression in *Xenopus laevis* oocytes seems to be well suited as no net zinc transport is observed at the latest stage of oocyte development [20]. Frog oocytes have been employed for measuring the zinc transport activity of ZnT5 [21]. Remarkably, despite being a vesicular transporter, we find ZnT8 proteins expressed in oocytes at the surface and active in zinc transport. Expression of ZnT8 isoforms in human embryonic kidney (HEK293) cells confirmed the differential sub-cellular localization of the two isoforms, indicating potential for differential physiological functions. This experimental system, therefore, is expected to provide critical information about the zinc transport activity of the class of vesicular zinc transporters (ZnT2-4), the zinc transport activity of which has not been investigated.

Materials and Methods

Plasmid construction

Genes of V5-tagged ZnT8 isoforms (R variant) were synthesized (Biomatik, Wilmington, DE, USA) and cloned in pcDNA3.1(+) into *Xho*I and *BamH*I restriction sites. The long isoform, N-V5-ZnT8lg, was designed to have the V5 tag inserted after the first 49 amino acids. The short isoform, V5-ZnT8sh, which lacks the first 49 amino acids, had the V5 tag introduced at the N-terminus. The tag was, therefore, in the same position in both isoforms. The W variants were obtained from the above-mentioned plasmids by site-directed mutagenesis (Mutagenex, Suwanee, GA, USA).

Expression in HEK293 cells

HEK293 cells were cultured in DMEM (Gibco, Carlsbad, CA, USA) containing 10% heat-inactivated FBS (Gibco), 100 U/ml penicillin (Gibco), and 100 µg/ml streptomycin (Gibco), at 37°C with 5% CO_2 in humidified atmosphere. Transient transfection of HEK293 cells with pcDNA3.1(+)-N-V5-ZnT8lg and pcDNA3.1(+)-V5-ZnT8sh was performed using FuGENE® HD (Promega, Madison, WI, USA) at a 3:1 ratio, according to manufacturer's instructions. Localization of tagged proteins was investigated 48 h after transfection.

Expression in X. laevis *oocytes*

Synthesis of RNA from pcDNA3.1(+) constructs linearized with *Eci*I was performed using the mMESSAGE mMACHINE/T7 RNA polymerase transcription kit (Ambion, Foster City, CA, USA) according to the manufacturer's instructions. Oocytes, originating from the European Xenopus Resource Centre (EXRC, Portsmouth, UK), were defolliculated in two 30-min incubations with 2 mg/mL collagenase (Sigma-Aldrich, St. Louis, MO, USA) in calcium-free Barth's Modified Saline (BMS; 88 mM NaCl, 1 mM KCl, 0.82 mM $MgSO_4$, 10 mM HEPES). Defolliculated oocytes were then washed in complete BMS [with calcium: 0.33 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$], and stage VI oocytes were selected. Selection was based on size and pigmentation of the poles and equatorial band. Oocytes were left for one day in complete BMS supplemented with 1 mM sodium pyruvate (GE Healthcare, Chalfont St. Giles, UK) and 50 µg/mL tetracycline (Sigma-Aldrich), and then injected with 18 ng of cRNA and incubated for 3 days in fresh medium before performing radiotracer efflux assays, immunolocalization, and protein extraction. Non-injected oocytes were used as controls.

Cell membrane biotinylation

Biotinylation of cell membrane proteins was adapted from Carvalho *et al*. [22]. Briefly, 20 oocytes were washed twice in PBS-glucose (1 mg/mL) and incubated, for 1 h on ice and with constant shaking, in 1 mL of 1 mg/mL EZ-Link™ Sulfo-NHS-SS-Biotin (Thermo Scientific, Boston, MA, USA) prepared in PBS-glucose. The reaction was stopped by the addition of 500 mM glycine, pH 7.4. Cells were washed three times with PBS-glucose and lysed in 100 µL homogenization buffer [100 mM NaCl, 1% (v/v) Triton-X100, 20 mM Tris-HCl, pH 7.5] with a Halt™ Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific) supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF). The extracts were diluted in 900 µL of PBS-glucose and incubated with 15 µL of streptavidin-bound Dynabeads® MyOne™ Streptavidin C1 (Invitrogen, Carlsbad, CA, USA), for 1 h in a rotating shaker, at 22°C. Beads were magnetically separated, washed four times in PBS-glucose, and resuspended in 30 µL of 50 mM 1,4-dithiothreitol (DTT) and 4 M urea prepared in Laemmli sample buffer. After 2 h of incubation at room temperature, proteins were run on an SDS gel and subjected to western blotting. For extraction of the total protein, 10 oocytes were lysed by continuous

pipetting in 50 mM Tris-HCl pH 7.4, containing a cocktail of protease inhibitors. The suspension was spun for 2 min at 300 ×g to remove yolk proteins, and the supernatant was recovered. Extracts were run on an SDS gel after addition of 4 M urea and 1% (v/v) β-mercaptoethanol in Laemmli sample buffer, without boiling or heating, and subjected to western blotting. Membranes from biotinylated and total extracts were hybridised with anti-V5 antibody (ab27671, Abcam, Cambridge, UK) and re-hybridised, after stripping, with anti-GAPDH, a cytosolic protein (mAbcam 9484, Abcam).

Indirect immunofluorescence assay

To localize ZnT8 isoforms expressed in *X. laevis* oocytes and in HEK293 cells, cells were first washed three times in PBS, fixed in 4% (w/v) paraformaldehyde and permeabilized with 0.1% (v/v) Triton X-100. After blocking with 1% (w/v) bovine serum albumin (BSA), cells were incubated with anti-V5 antibody overnight, followed by Alexa Fluor® 594-conjugated antibody (Molecular Probes, Carlsbad, CA, USA) for 1 h. In HEK293 cells, DNA was stained with 2-(4-amidinophenyl)-1H-indole-6-carboxamidine (DAPI), and the coverslips were then mounted in 70% (v/v) glycerol in PBS. Images of oocytes were acquired with an A1R Si MP Confocal Microscope (Nikon, Tokyo, Japan), and images from HEK cells were acquired with a Leica Confocal Microscope (Leica Microsystems, Wetzlar, Germany).

^{65}Zn efflux assays in X. laevis *oocytes*

Zinc efflux in oocytes was measured after loading the cells with ^{65}Zn (specific activity = 75.3 MBq/mg; Polatom, Otwock, Poland). Each oocyte was placed in a tube containing 500 µL of efflux buffer. After 1 h at 19°C, the buffer was transferred to another tube, the oocyte washed with 500 µL of efflux buffer, and the medium recovered in the same tube as before. The radioactivity in the medium and that associated with the oocyte were measured in a γ-counter (Beckman Coulter, Fullerton, CA, USA). Results are given as the percentage of counts per minute (cpm) of ^{65}Zn in the supernatant in relation to the total cpm of injected ^{65}Zn per oocyte (sum of the radioactivity in the oocyte and in the supernatant). Five control or cRNA-injected oocytes were used per condition. The conditions tested in the efflux assays are shown in Table I.

Statistical analysis

Data were analysed using SPSS 20.0 (IBM Corp., Armonk, NY, USA). Each efflux experiment was expressed as the mean value and standard deviation for descriptive analysis. Data distribution was tested with the Shapiro-Wilk test. One-way ANOVA or the Mann–Whitney test was used for normal distribution and non-normal distribution of variables. Bonferroni or Tamhane multiple comparison post hoc tests were used, after one-way ANOVA, depending on the Levene's test for the homogeneity of variances. The level of significance was set at 0.05.

Results

CDF members are present in Archaea, Eubacteria, and Eukaryotes, and all functionally characterised CDF proteins are Me^{2+}/H^{+} or Me^{2+}/K^{+} antiporters, transporting divalent metal ions from the cytosol to the lumen of organelles or to the exterior of the cell. In order to identify amino acids that are characteristic of vesicular transporters and for ZnT8, we performed a sequence alignment. Alignment of amino acidic sequences of ZnT8 from several vertebrate species and comparison with the sequence of YiiP from *E. coli* (also known as ferrous iron and zinc transporter, Fief) reveals a high degree of conservation in critical parts of the molecule (Fig. 1A). All of the sequences, except those from the frog, present the CDF family signature proposed by Montanini *et al.* [23] and derived from the multiple sequence alignment of characterized and hypothetical CDF members: [SC]-X-[ASG]-[LIVM]-[LIVMTAF]-[SATG]-[DAELSTY]-[SGALFMTV]-{DKNPQR}-[HDNEL]-X_3-[DH]-X_2-[ASGLNTI]-$X_{20–25}$-G-X_2-[KNQRSY]-X-[DEGLNPQRST], where X is any amino acid, any one of the amino acids shown within square brackets is possible, and none of the amino acids within braces are possible. Humans and rats have two ZnT8 isoforms. The amino acid sequences of the human ZnT8 isoforms are 100% identical except that one (Q8IWU4_1) possesses an N-terminus that is 49 amino acids longer; the rat isoforms also are 100% identical except for the last two amino acids and for the fact that one isoform has a C-terminus that is 46 amino acids longer. With the exception of *X. tropicalis*, which is predicted to have two isoforms as well, albeit showing some sequence variation, only one protein is predicted in all other species. The

amino acids important for CDF function in non-human species are all present in human ZnT8 (Fig. 1A). Sequences from mammals also present the di-leucine motif [DE]-X_3-L-[LI] (aa 269-274) in the C-terminus.

As expected, the regions with the highest degree of identity and similarity are the transmembrane domains (TMDs). Nevertheless, the N-termini show conservation and similarity of some amino acids among the animal ZnT8 sequences, and the region downstream of the last TMD, from amino acids 270 to 321 in the C-terminal domain, is rather highly conserved but not from amino acids 322 to 344. Additionally, there is high similarity among the ZnT8 sequences of mammals (humans, chimpanzees, rats, mice, and dogs) not only in the N-terminus but also in the region between amino acids 322 and 344. This grouping is in agreement with the phylogenetic tree where mammals separate from amphibians, birds, fish, and reptiles, and the *E. coli* sequence separates from all others (Fig. 1B).

Localization of V5-tagged ZnT8 in HEK293 cells

Despite being expressed mainly in the pancreatic α- and β-cells, *ZNT8* transcripts have also been detected in the kidneys and testes [4, 9]. To determine the localization of the two ZnT8 isoforms, we designed two synthetic genes from the W325 variant: one in which the V5 tag was inserted after the first 49 amino acids (N-V5-ZnT8lg) and the other lacking the first 49 amino acids and carrying the V5 tag at the N-terminus (V5-ZnT8sh) (Fig. 2). Therefore, both genes were tagged at the same position; the only difference being that one coded for a protein that is 49 amino acids shorter. The long forms carrying one of several different N-terminal tags were also tested but none of these produced a detectable protein, suggesting that ZnT8 is N-terminally cleaved. When HEK293 were transfected with plasmids carrying either one of the two forms the majority of cells transfected with the long isoform showed fluorescence at the cell membrane as well as in intracellular membranes (Fig. 3A), whereas the short isoform was observed primarily in intracellular membranes (Fig. 3B).

Expression of ZnT8 isoforms in X. laevis *oocytes*

The *X. laevis* oocyte system was used to compare zinc transport activity of the two isoforms of human ZnT8. In order to measure zinc transport across the plasma membrane, these proteins are required to be expressed at the surface of the cell. Oocytes were injected with cRNA produced from the W325 variant of the N-V5-ZnT8lg and V5-ZnT8sh genes, and the expression and location of the proteins were assessed by immunofluorescence and western blotting of biotinylated cell-surface proteins. Non-injected oocytes were used as controls. Confocal imaging of whole cells demonstrated that both ZnT8 isoforms are expressed in the plasma membrane (Fig. 4A). In some cells, a double ring was observed, which could be due to ZnT8 in the cell membrane and to the high level of fluorescence of the yolk [24] and/or ZnT8 expression in intracellular membranes such as the ER that can contact the plasma membrane. Given the limiting penetration depth of the laser, we could detect only signals a few micrometers below the cell surface. Any expression in deeper structures will remain unidentified by this method. The presence of ZnT8 at the cell membrane of oocytes was confirmed by cell-surface biotinylation. Western blot of isolated plasma membrane clearly shows that both isoforms of ZnT8 are present at the surface of the oocytes (Fig. 4B). The higher molecular mass bands are consistent with ZnT8 dimers. As control for possible intracellular biotinylation, we used the cytosolic protein glyceraldehyde phosphate dehydrogenase (GAPDH). GAPDH was not detected in the biotinylated fraction, demonstrating that biotinylation occurred only at the cell membrane. Given that the number of oocytes used in the biotinylation protocol was twice that used in total protein extracts and that the intensity of the bands is lower in the biotinylated fraction than in the total extracts, it can be concluded that, although ZnT8 is present at the surface of oocytes, the majority of ZnT8 is located in intracellular membranes. Thus, the net export observed in the following transport assay indicates that the capacity of all intracellular zinc stores is already exhausted.

Radiotracer transport assays in oocytes

ZnT8 is expected to transport metal ions away from the cytosol and, therefore, out of the cell when present at the cell membrane. Therefore, the assays in *X. laevis* oocytes focused on the analysis of ^{65}Zn efflux from oocytes. Radiotracer transport assays were

performed three days after nano-injection of cRNA. Oocytes were loaded with ^{65}Zn, and the amount of radioactivity in the supernatant assessed after 1 h.

Among all the conditions tested, the conditions for reproducible results are: injection of ^{65}Zn together with 10 mM nitrilotriacetic acid (NTA; a zinc chelator), at a pH value higher than 4, and an extracellular buffer without calcium and with 1 mM NTA, at pH 7.4. Loading of oocytes with ^{65}Zn-pyrithione, instead of injecting ^{65}Zn, was ineffective and resulted in levels of extruded radioactivity that were close to the background of the γ-counter. Regarding the amount of ^{65}Zn injected, although the differences are not statistically significant, there was a trend to have higher levels of effluxed radioactivity when lower amounts of radionuclide were used.

Regardless of whether the radioactivity was counted in the efflux buffer or in the oocytes, analysis of the experiments showed statistically significant ^{65}Zn transport activity of the two isoforms but no statistically significant difference in transport activity between the two isoforms (Fig. 5). Preliminary transport activity data also showed no difference between W and R variants.

Discussion and Conclusion

We demonstrate that the long isoform of ZnT8, the one generally used for exogenous expression studies of ZnT8, is not only expressed in vesicles but also to a high extent in the plasma membrane of HEK293 cells. In contrast to this finding, the long isoforms of ZnT2 and ZnT5 were detected in internal membranes and the shorter ones in the cell membrane [21, 25-28]. Given that we expressed ZnT8 from vectors, it cannot be excluded that the long form was mislocalised due to overexpression; nevertheless, the cytolocation of the short form expressed in the same manner was mainly intracellular, arguing against such an interpretation of the results. We presently do not know if plasma membrane-located ZnT8 is exporting zinc from β-cells. However, both isoforms of the protein locate to the cell surface when expressed in *X. laevis* oocytes where they efflux zinc. Other human ZnTs also present different isoforms that are either at the plasma membrane or in the membrane of organelles, as is the case for ZnT2 and ZnT5 (Table II). Zinc transporters 3, 4, and 7 are intracellular transporters and do not have isoforms; ZnT5, 6, and 10 present several possible

isoforms, but experimental information on the cellular localization does not exist for some of them.

Human ZnT8 possesses the amino acids that have been shown, by site directed mutagenesis, to be important for the function of several CDF members in species such as *E. coli*, *Cupriavidus metallidurans*, and *Populus trichocarpa* x *Populus deltoides* [23], namely H52, 54, 106, 220, 304, and 345, S57 and 228, E88 and 276, D103, 110, 224, and 248, and G135. According to the classification considering the residues conserved in TMDs II and V and the metal ion being transported [23], human ZnT8 clusters with Zn-transporting CDFs. Other characteristics of Zn-CDF transporters are also present in human ZnT8: N- and C-termini rich in histidine and serine residues and histidines in the region between TMDs IV and V, the latter ones might be involved in zinc-binding [29, 30]. Remarkably, there is neither conservation of the amino acid at position 325 nor of its properties (*i.e.*, hydrophobicity, charge, ability to form H-bonds, and presence of aromatic rings): R for human (or W), chimpanzee and dog, Q for mouse and rat (for one of the isoforms; the other is only 322-amino acids long), V for chicken, zebrafish, and *E. coli*, G for frog, and A for anole, a lizard. Additionally, in frog ZnT8 sequences, the zinc ligands in TDMII [31, 32], corresponding to positions 106 and 110 (human ZnT8 numbering), are not conserved with the exception of histidine 106 in the second isoform, F6T4F9. The di-leucine motif in the C-terminus of mammalian ZnT8 sequences, usually associated with clathrin-coated vesicle-mediated endocytosis, might be required for the endocytosis of ZnT8 present at the surface of the cell following membrane fusion of insulin dense granules (vesicles) and secretion of insulin and zinc.

While two isoforms have been noted for humans, rats, and frogs, there is a lack of experimental evidence for other species, either from RNA or protein sequencing data. With the exception of the human and rat longer isoforms, ZnT8 from all the species used in the alignment are annotated in the Ensembl database as *predicted* proteins. It needs to be seen if ZnT8 is expressed as two isoforms in species from different animal classes.

Not all mRNA molecules in a cell are translated at a given time; therefore, the existence of *ZnT8* transcripts encoding two different isoforms does not mean that the cell expresses both proteins. The *ZnT8* alternative transcripts were detected in an RNAseq analysis of human pancreatic islets [13]: the most abundant transcript (71.3%) encodes the

longer protein (Q8IWU4_1), while the other (28.7%) encodes the 49-aa shorter one (Q8IWU4_2). Current knowledge does not allow conclusions as to whether both or only one isoform is expressed.

Transport activity of human ZnT8 has been investigated employing mainly the longer isoform. These investigations, using either ^{65}Zn and isolated β-cell vesicles [17] or fluorescent probes and cell lines [7], showed that the R325 variant is less active than the W counterpart. However, recently, using a fluorescence-based assay in proteoliposomes, the R variant of the shorter isoform turned out to be more active than the W variant [18]. Results are, therefore, controversial, or the use of different isoforms might account for the differences. The *Xenopus* oocyte transport assay presents a convenient and well-controlled system to study different forms and variants of ZnT8 under exactly the same conditions. A few pilot experiments performed in the present study could not detect a difference between the two variants, perhaps in agreement with the observation that the type of lipid environment is a factor in revealing differences in transport in proteoliposomes [18].

Uptake and efflux transporters are widely investigated proteins. In the pharmaceutical industry, they are important targets for drug design and for understanding transport-mediated uptake of drugs and mechanisms of drug resistance. Properties of metal transporters have been investigated using a number of systems such as functional complementation of *Saccharomyces cerevisiae* and transport assays in *X. laevis* oocytes, in isolated organelles, in monolayers of cell lines, and in *in vitro* synthesized lipid vesicles containing reconstituted recombinant transporter. Although useful, all these systems have drawbacks, such as incorrect protein folding and ambiguity in orientation in the membrane after reconstitution in liposomes, lack of endogenous interacting proteins, time-consuming and expensive preparation of proteoliposomes, variability of the quality of oocytes, and contribution of endogenous transporters. In the specific case of human ZnT8, some of these systems have been used, namely assays in β-cell lines, in isolated vesicles, and in proteoliposomes. The assays in β-cells are the ones that have an environment closest to the native one. However, it is not possible to control every condition in the cell, such as its metabolic state and zinc concentration, for instance. Furthermore, zinc transport by variants and isoforms introduced into the cell will take place against a background of endogenous ZnT8, unless conducted in a cell line where no endogenous ZnT8 is expressed. Transport

assays with vesicles isolated from β-cells are a robust experimental system but are limited by the amount of material one can obtain as well as background expression of endogenous ZnT8. Proteoliposomes can be employed to test various assay conditions, but they constitute a very simple system where interacting proteins are missing. In contrast, the *X. laevis* oocyte expression system has not been employed previously to investigate the function of ZnT8, though it has been widely used for studies of other transmembrane solute transporters. It became possible to employ it for human ZnT8 because we found the protein to be expressed at the cell membrane of oocytes. Additionally, the dimers observed in western blots suggest that, in oocytes, both ZnT8 isoforms maintain a requisite condition for metal transport activity: dimerization. Particularly important for this work is the fact that oocytes do not express many endogenous membrane transporters and channels in the latest stage of development [24, 33]. These characteristics make *X. laevis* oocytes an attractive system to study functional properties of heterologously expressed transporters. While *X. laevis* oocytes go through six maturation stages, their zinc content increases gradually as the egg yolk protein, vitellogenin, incorporates zinc [34], and the zinc content remains constant from stage VI of oogenesis until at least stage III of embryogenesis [35]. Therefore, stage VI oocytes are a closed system concerning zinc exchange with the environment, and neither zinc efflux nor influx is expected to occur through oocyte-specific zinc transporting systems and to affect the assay of a heterologously expressed zinc transporter.

We faced significant variability throughout the assays, which is often observed in experiments performed in *X. laevis* oocytes. The quality of oocytes has been observed to undergo seasonal variations and also to vary within a batch of oocytes derived from either the same frog or from different frogs [36, 37]. Intra- and inter-experimental variability is also affected by the maturation stage of the oocytes as selection depends on visual inspection of the eggs. Although the variability among the experiments was high, the results suggest that loading of the oocytes is better performed by injecting ^{65}Zn together with NTA, which keeps the metal ion in solution. Loading of oocytes with ^{65}Zn using pyrithione takes longer time than by injection and, therefore, increases the chances of zinc being compartmentalised within the oocytes and less available for efflux by ZnT8. Concerning the tested amounts of injected ^{65}Zn (442, 663, or 995 fmol), the assays show better results when the lower amounts are used. Considering that oocytes in stage VI of maturation have 70 ng of zinc [37],

these cells have about 1000 fmol of zinc; therefore, injection of 442 fmol of zinc increases the intracellular concentration of this metal by nearly 50%, and injection of 995 fmol, almost doubling and potentially generating considerable stress to the oocyte. The injection of a lower concentration of ^{65}Zn could yield better results. For example, when studying iron export by ferroportin, injection of a lower concentration of ^{55}Fe led to an increased efflux [38]. Injecting less ^{65}Zn to investigate ZnT8 was not possible as our experiments were limited by how much ^{65}Zn could be detected by the γ-counter. Regarding the pH of the injected zinc solution, a higher pH value promoted more ^{65}Zn transport across the oocyte membrane towards the extracellular medium. Also, an efflux buffer without calcium and with NTA at pH 7.4 is the best condition. Extracellular NTA could be acting as an acceptor akin to insulin in the granules, generating an outward concentration gradient. A similar observation was made by others [38]: a higher efflux of iron was recorded when apo-transferrin and iron chelators were present in the efflux buffer. Concerning the pH value of the extracellular medium, given that the intracellular pH of the oocyte is also approximately 7.4 [39] and different concentrations of K^+ in the buffer did not produce differences in ^{65}Zn efflux, we hypothesize that a local increase in proton concentrations is responsible for driving zinc ions in the opposite direction, into the extracellular medium. *X. laevis* oocytes have a Na^+/H^+ exchanger at their membrane, which could fulfil such a function [40].

ZnT8 is one of the four islet autoantigens. While its presence on the plasma membrane increases when insulin granules fuse with the β-cell plasma membrane during glucose induced insulin secretion (GIIS) [12], our findings provide an alternative explanation for its presentation at the cell surface. They demonstrate a substantial amount of ZnT8 at the plasma membrane in the absence of GIIS. Examination of the N-terminus of the long form of ZnT8 with the ELM database predicts ERTYLV (amino acids 5 to 10) as an endosome-lysosome-basolateral sorting signal (ELME000149) that removes proteins from the plasma membrane (Nathan Williams, Dissertation for an MSci degree at King's College London 2015). Two types of sorting signals have been described, one of which relates to a conditional presence of the protein at the plasma membrane because removal depends on a phosphorylation event [41]. The presence of a threonine as well as a tyrosine in the ERTYLV sequence suggests that removal of ZnT8 from the plasma membrane depends on the action of a yet to be identified kinase.

Conflict of interest

The authors declare no conflict of interest.

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Figure Legends

Figure 1 – Alignment of the amino acid sequence of human ZnT8 with those of other animals and *E. coli* Yiip **(A)** and phylogenetic tree **(B)**. **A)** ZnT8 sequences of amino acids that have been shown to be functionally important [23] are denoted with an asterisk (*). Dashed line shows the location of the CDF family signature sequence. Grey shaded boxes in TMDs II and V highlight the conserved motifs found in Zn-CDF members. Amino acid numbering and transmembrane domains (as predicted in the UniProt database) are shown in relation to the long isoform of human ZnT8 (Human_1). **B)** Phylogenetic tree with branch distances. Sequences were retrieved from UniProt database. Human_1 and _2 (*Homo sapiens*): Q8IWU4_1 and Q8IWU4_2; Dog (*Canis lupus familiaris*): F1PTQ3; Mouse (*Mus musculus*): Q8BGG0; Chimpanzee (*Pan troglodytes*): H2QWL9; Rat_1 and _2 (*Rattus norvegicus*): P0CE46 and A0A0H2UHD4; Anole (*Anolis carolinensis*): G1KKM1; Chicken (*Gallus gallus*): A0A1D5NY81; Zebrafish (*Danio rerio*): A0A0R4IFM6; Frog_1 and _2 (*X. tropicalis*): F6W4S9 and F6T4F9; *E. coli* Yiip: P69380. The alignment and the phylogenetic tree were performed using Clustal Omega. The output of the alignment was visualised by ESPript 3.0 [42]. Amino acids conserved in all sequences at a given position are shaded in black. The consensus sequence is given: uppercase letters show identity (consensus in 100% of the sequences); lowercase letter show consensus in more than 50% of the sequences; **!** is any of IV; **$** is any of LM, and **#** is any of NDQEBZ.

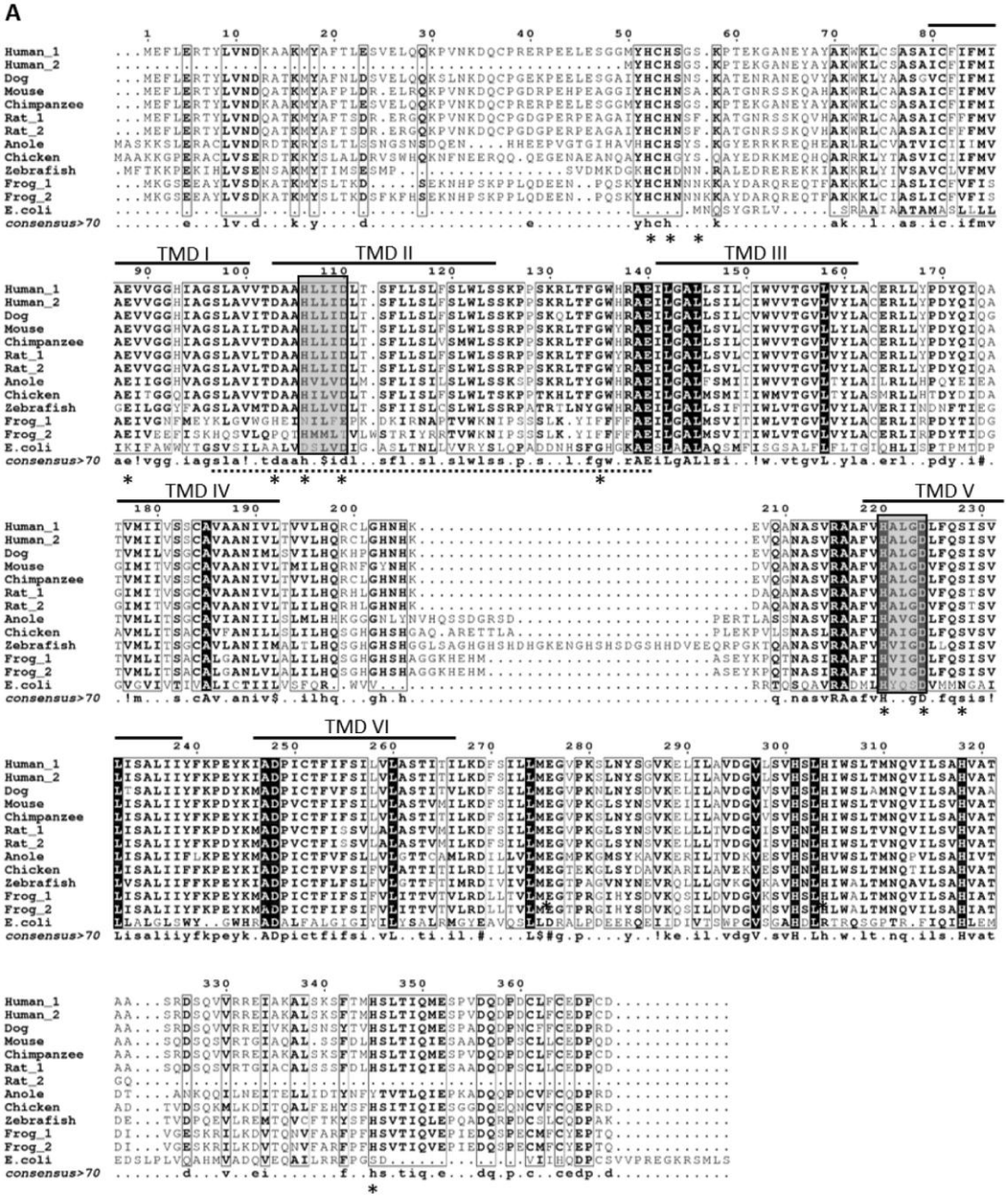
Figure 2 - Scheme of the genes cloned into pcDNA3.1(+). In the N-V5-ZnT8lg genes, the V5 tag was placed after the first 49 amino acids. In both W and R variants of the short isoform, the tag was placed in the N-terminus, in the exact same position as in the long isoform.

Figure 3 – Localization of human ZnT8 isoforms (W325 variant) in HEK293 cells. HEK293 cells were transfected with vectors carrying the N-V5-ZnT8lg and the V5-ZnT8sh genes. Proteins were detected with anti-V5 tag antibody and the nuclei stained with DAPI. Representative experiment out of n=4.

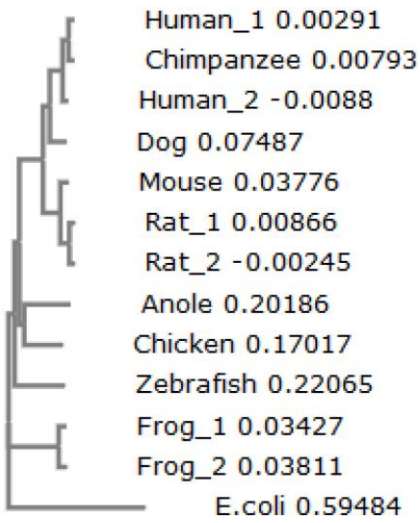
Figure 4 – Localization of human ZnT8 variants and isoforms in *X. laevis* oocytes. **A and B)** ZnT8 variants and isoforms are expressed at the surface of oocytes. *Xenopus laevis* oocytes were injected with cRNA for the isoforms and variants of human ZnT8, and localization was examined by **(A)** laser confocal microscopy in whole cells and by **(B)** western blotting of total

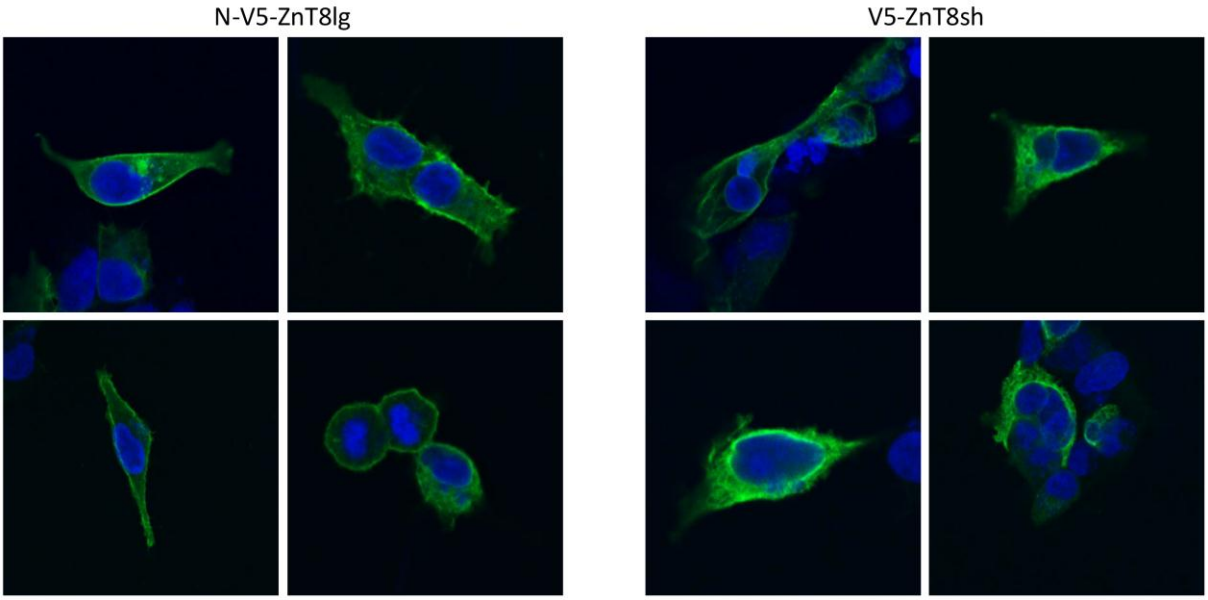
(T) and cell-surface biotinylated (B) proteins. ZnT8 proteins were detected with anti-V5 tag antibody, and the specificity of biotinylation to cell surface proteins was controlled, upon stripping of the membrane, with an antibody against cytosolic GAPDH. Arrow heads indicate ZnT8-specific bands. Representative experiment out of n=3.

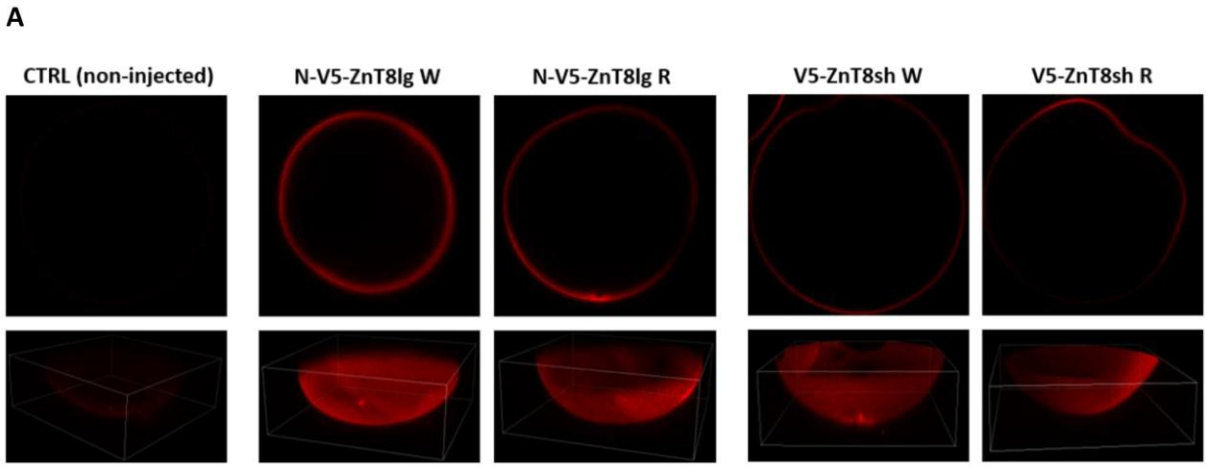
Figure 5 – ^{65}Zinc transport assay. Oocytes were co-injected with ^{65}Zn and 10 mM NTA, placed for 1 h in efflux buffer containing 1 mM NTA, at pH 7.4, and the radioactivity in the extracellular medium was measured in a γ–counter. Mean ± S.D.; n=4; ***p<0.005.

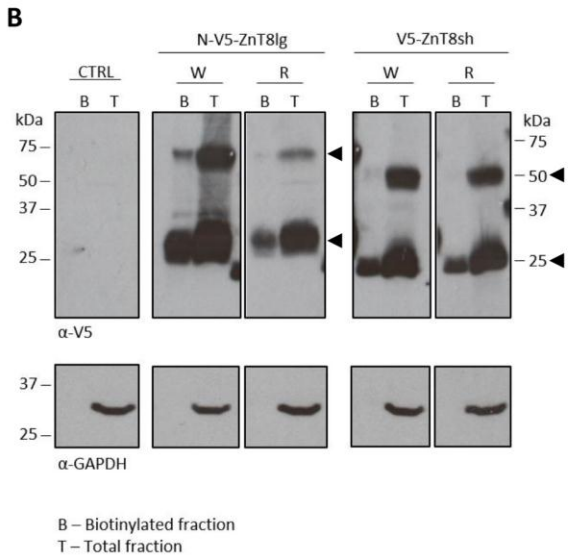


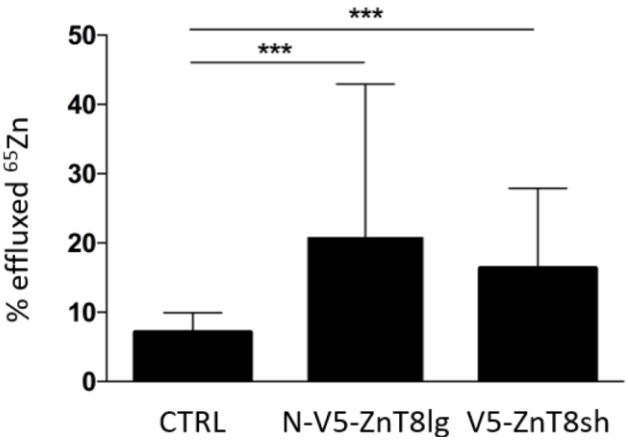
B











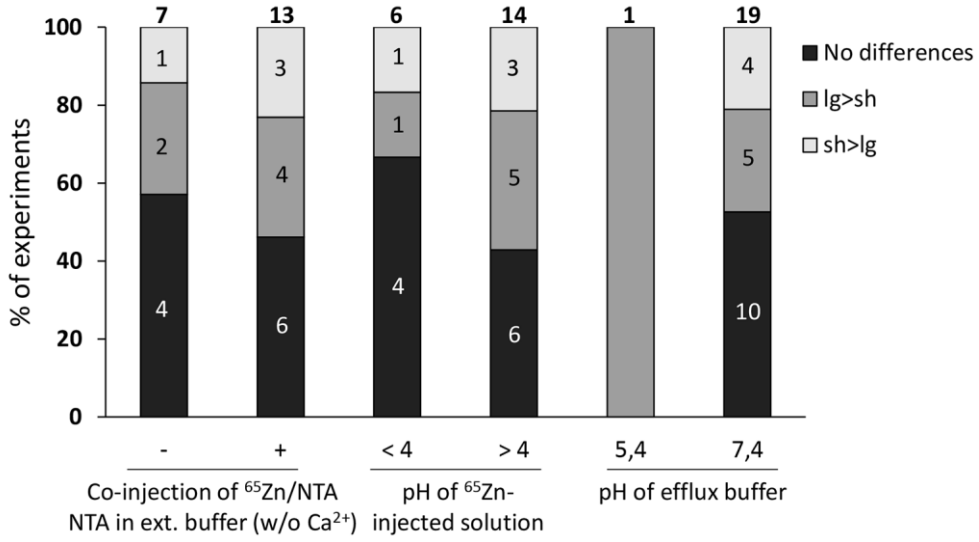


Table I – Conditions tested in ^{65}Zn efflux assays.

Conditions tested
Loading of oocytes with ^{65}Zn by nano-injection or with 20 µM pyrithione and 50 µM ^{65}Zn
Injection of 442, 663, or 995 fmol ^{65}Zn
Injection of ^{65}Zn at pH 1.1, 3.3, 5.5, or 7
Injection of ^{65}Zn with 10 mM NTA
Injection of 9, 18, or 25 ng of *N-V5-ZnT8lg* or *V5-ZnT8sh* cRNA Presence of 1 mM NTA or 0.1% BSA in the efflux medium, without Ca^{2+}
Efflux buffer at pH 5.4 or 7
Presence of 1, 2.5, or 5 mM K^+ in the efflux buffer
Presence of pyruvate or of bicarbonate in the culture medium
Pre-culture of oocytes with 0.25, 1, or 5 mM pyruvate

Table II – Isoforms and cellular localization of ZnTs. Information was retrieved from human ZnTs in the Ensembl database, and only transcripts supported by at least one non-suspect mRNA were addressed. Highlighted in grey are data arising from evidence only at the transcript level, lacking experimental evidence, or that have not been manually annotated and reviewed by UniProtKB curators. Data on the localization of human ZnTs was reviewed by Kambe *et al*. [2], with the exception of ZnT8, which is addressed in this study and by Huang *et al*. [12].

ZnT	Isoforms (UniProt)	Predicted N-terminal length (aa) and number of TMDs	Protein length (aa)	Location in the cell
ZnT1	1 (Q9Y6M5)	9 (6 TMDs)	507	cell membrane
ZnT2	2 (Q9BRI3)	90 (5 TMDs)	323	cell membrane
		139 (5 TMDs)	372	internal membranes
ZnT3	1 (Q99726)	74 (6 TMDs)	388	internal membranes
ZnT4	1 (O14863)	112 (6 TMDs)	429	internal membranes
ZnT5	4 (Q8TAD4)	32 (16 TMDs)	765	internal membranes
		22 (11 TMDs)	523	cell + internal membranes
		32 (2-3 TMDs)	118	unknown
		0 (0 TMDs)	77	unknown
ZnT6	3 (Q6NXT4)	32 (6 TMDs)	461	Internal membranes
		32 (5-6 TMDs)	501	unknown
		32 (6 TMDs)	438	
		4 (6 TMDs)	432	
	1 (B5MCR8)	26 (4 TMDs)	387	
ZnT7	1 (Q8NEW0)	36 (6 TMDs)	376	internal membranes
ZnT8	2 (Q8IWU4)	30 (6 TMDs)	320	internal membranes
		79 (6 TMDs)	369	cell + internal membranes
ZnT9	1 (Q6PML9)	238 (5 TMDS)	568	internal membranes

ZnT10	3 (Q6XR72)	10 (6 TMDs)	485	cell + internal membranes
		0 (2 TMDs)	240	unknown
		10 (4 TMDs)	223	unknown